Immunohistochemical Analysis of Cerebella in Mice With Conditional Depletion of IKKβ in the Microglia

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Abstract:

Spinocerebellar Ataxia Type-1 (SCA-1) is an inherited neurodegenerative of the cerebellum caused by a mutant CAG polyglutamine repeat tract in the portion of the *Sca1* gene that codes for the protein Ataxin-1. SCA-1 is frequently studied in mouse models. In almost any case of neurological injury, hypertrophy and proliferation of glial cells, called gliosis, is observed. Gliosis has potentially harmful effects on the pathology of neurological degeneration. In order to the effect of cytokine-mediated gliosis on SCA-1 mice, Lys-M-Cre IKKβ^{Flox} transgenic mice were created. These mice have conditional depletion of IKKβ in the cerebellar microglia, IKKβ is an enzyme necessary for NF-κB mediated cytokine production, and without it, cells do not produce pro-inflammatory cytokines. We examined the cerebella of Lys-M-Cre IKKβ^{Flox} mice through immunohistochemical staining, using microglia-specific marker IBA-1, astrocyte-specific markers GFAP and S-100, and Purkinje neuron-specific Calbindin. From IBA-1 analysis, we have found that the loss of IKKβ does not cause microglial activation. Calbindin analysis revealed that the cerebella of Lys-M-Cre IKKβ^{Flox} mice are morphologically similar and comparable to those that express IKKβ. GFAP and S-100 analysis demonstrated results in the astrocytes opposite of IKKβ depletion in microglia, which merits further analysis.

Introduction:

Spinocerebellar Ataxia Type-1 (SCA1) is a neurodegenerative disease characterized by progressive degeneration of the cerebellar Purkinje neurons resulting in impaired coordination, impaired cognition, and eventual death due to respiratory failure. SCA-1 results from an expansion of the CAG repeat tract in the portion of the *Sca1* gene which codes for the protein Ataxin-1 (ATXN1)(Cummings et al., 1998). The expansion of the poly-glutamine repeat tract in the ATXN1 protein is thought to cause a gain-of-function mutation, where the onset of symptoms starts earlier and becomes more pronounced with more glutamine repeats (Burright et al., 1995; Clark et al., 1997). The Cvetanovic Lab studies SCA1 in mouse models of the disease, namely ATXN1[81Q], which has 81 copies of glutamate in the coding region for the ATXN1 protein, selectively expressed in the Purkinje neurons. One of the aims of the Cvetanovic lab is to investigate the role of glial cells in SCA1 pathology in these mice.

Neurological damage, such as that observed in SCA-1, is almost always paired with inflammation and activation of glial cells surrounding damaged neurons, especially astrocytes and microglia (Burright et al., 1995). Gliosis is a complex response marked by glial hypertrophy, proliferation, and in severe cases, scarring. Glial cells secrete pro-survival factors during gliosis to maintain the optimal extracellular environment for neurons in the event of neurological injury, and have been shown to have key roles in various neurodegenerative disorders. Ablating glial cells following physical neurological trauma in mice resulted in increased neurological damage as compared to mice in which glial ablation was not performed (Sofroniew, 2009). Despite the

protective effects of gliosis, the cytokine-mediated inflammation response expressed by microglia and astrocytes is potentially damaging; Individuals who have experienced a markedly higher cytokine-mediated neurological activation in their lifetime are pre-disposed to a variety neurodegenerative disorders. For example, the incidence of dementia among Spanish-Flu survivors is higher compared to their peers (Deleidi & Isacson, 2012). These studies confirm that while glial cells have a key neuro-protective function, inflammatory cytokines produced by glial cells can aggravate neurological damage. Previous research in the Cvetanovic lab has demonstrated that gliosis occurs early in SCA1, so it is possible that glial cells contribute to SCA1 pathology as well. Consequently, the Cvetanovic Lab is studying the effects of eliminating just the cytokine-mediated component of gliosis in SCA1 pathology. This study examined the efficacy of a transgenic modification aimed at depleting the cytokine-mediated inflammation pathway in the cerebellar microglia of non-SCA1 mice, to see if this modification could demonstrate reduced microglial activation. By using a variety of immunohistochemical stains on pre-prepared whole-brain sections of the mice, cerebellar microglial activation could be determined. Understanding the impact of this modification on healthy mice will allow us to apply the same modification on a mouse model of SCA1, so that we can explore the impact of depleting cytokine-mediated gliosis on SCA1 pathology.

Methods:

This study examined the brains of transgenic mice prepared and frozen by the Cvetanovic Lab. Sections taken from each brain were immunohistochemically stained using Purkinje-neuron specific anti-Calbindin antibodies, anti-Glial Fibrillary Acidic Protein (GFAP) antibodies, anti-S100 (β) antibodies, and anti-Ionized Calcium Binding adaptor molecule 1 (IBA-1) antibodies. Calbindin proteins are prominent in the Purkinje neurons of the cerebellum (Maeda, Ellis-Davies, Ito, Miyashita, & Kasai, 1999), so Purkinje cell-specific Calbindin binding antibodies are used to examine the dendritic morphology of the Purkinje neurons. GFAP and S-100β proteins are highly expressed in activated astrocytes, and these two antibodies are used to monitor the activation of the cerebellar astrocytes (Esposito et al., 2006)(Brahmachari, Fung, & Pahan, 2006). IBA-1 is highly expressed in the cytosol of microglia, so IBA-1 binding antibodies are used to monitor microglial activation (Ahmed et al., 2007). By imaging sections stained with the aforementioned antibodies using a confocal microscope, these sections could be analyzed for changes in glial activation and dendritic morphology, as revealed by the antibodies used.

The transgenic modification in the experimental mice aims to conditionally disrupt the NF-κB cytokine-transcription pathway. Activation of NF-κB initiates the transcription of a critical majority of pro-inflammatory cytokines. The protein IκBα binds to NF-κB in the cytosol, inactivating it, until the enzyme IKKβ phosphorylates IκBα, freeing NF-κB to stimulate the production of cytokines. Using Cre-lox recombination (Strachan, 1999), the gene coding for IKKβ can be conditionally deleted to disrupt NF-κB-mediated cytokine production in a cell-specific manner. In Cre-lox recombination, a gene coding for the enzyme Cre-recombinase, accompanied by a variable promoter, is inserted into the genome of the host. In addition, loxP sites are inserted, flanking the target gene, which is to be interrupted (floxing). To create the transgenic mice used in this experiment, the promoter Lys-M was used to activate Cre recombinase in the cerebellar microglia only, and exon 3 of the gene coding for IKKβ was floxed. This creates Lys-M-Cre $IKK\beta^{Flox}$ mice, which have conditional depletion of IKKβ in the microglia. The control mice were IKKβ Flox Lys-M-Cre-mice, which do not have the Lys-M-Cre promoter.

Prior to the experiment, the Cvetanovic lab had prepared four whole brains taken from IKKβ Flox Lys-M-Cre- and Lys-M-Cre IKKβ^{Flox} mice. To do this, the mice were euthanized and their brains were extracted and fixed in 4% paraformaldehyde. Following fixation, the brains were cyroprotected in 30% sucrose, suspended in Tissue-Tek OCT compound and frozen at -80° C for storage.

Whole-brain midsagittal sections were taken from each of the brains. Sectioning was performed in a Leica cryostat sectioning machine at -20ºC. Fourteen slices, forty microns thick each, were taken from every brain, and then suspended in phosphate-buffered saline (PBS) for immediate staining.

Two sets of immunohistochemical staining were done. The first set used 6 brains (4 Lys-M-IKKβ^{Flox}, 2 IKKβFlox Lys-M-Cre-) stained with S100 (stain series 1). The second set used 4 brains (2 Lys-M-IKKβ^{Flox}, 2 IKKβFlox Lys-M-Cre-) stained with GFAP, calbindin, and IBA-1 (stain series 2). In Stain Series 2, two brains, one from each genotype, were cut significantly deeper into the midsagittal plane than the other two. The sections were washed three times with PBS to remove OCT media, and then blocked using a blocking buffer containing PBST (1% Triton–X detergent in PBS) and Normal Goat Serum (3%). Sections were then incubated in primary antibodies dissolved in blocking buffer (1:200 ratio stock antibody solution to blocking buffer). All sections in stain series 1 were treated with anti-S100 antibody (Sigma Aldrich). For stain series 2, half of the sections for each brain were treated with anti-IBA-1 (WAKO) rabbit antibodies, and the other half of the sections were treated with anti-glial fibrillary acidic protein (GFAP) rabbit antibodies (Sigma Aldrich), and anti-Calbindin-D-28K mouse antibodies (Sigma Aldrich). After 3 washes in PBST, sections were incubated in with a fluorescent antibody solution (1:400 ratio stock antibody solution to blocking buffer). For IBA-1 sections we used Alexa-fluor 594 goat anti-rabbit antibodies, while GFAP and Calbindin sections were treated with Alexa-fluor 594 goat anti-rabbit and Alexa-fluor 488 goat anti-mouse antibodies. Stained sections were then mounted onto glass slides, dried, and covered using Vector Vecta-Shield hard-set mounting medium with DAPI.

Imaging was performed using an Olympus FV1000 confocal microscope and Olympus Fluo-View software. Six images measuring 640x640 microns in area each were taken of the cerebellar lobules at 20X. Images consisted of stacks of 1-micron thick layers. Images of GFAP and calbindin were 22 layers per stack, while images of IBA-1 consisted of 23 layers per stack.

Images were analyzed using Fiji/ImageJ2 software. For staining series 1 (S100), six brains were analyzed, two IKKβ Flox Lys-M-Cre-and four Lys-M-Cre IKKβ^{Flox}. For staining series 2 (GFAP, Calbindin, and IBA-1), four brains, two IKKβ Flox Lys-M-Cre-and two Lys-M-Cre IKKβ^{Flox} mice were analyzed. Six images were analyzed per brain, and for every image; the full image stack was analyzed at once. For S100, the intensity of two randomly selected square areas encompassing the molecular and granular layers was measured. Average S100 intensities were then compared between brains. For GFAP, the intensities of two randomly selected square areas encompassing the white matter as well as two square areas encompassing the molecular and granular layers were. Average GFAP intensities for both the white matter as well as the granular and molecular layers were then compared between brains. For Calbindin and stained brains, the length of two randomly draw lines, perpendicularly stretching from the inner edge to the outer edge of the molecular layers, were measured in each brain. Average width of the molecular layer was then compared between brains. Average Calbindin staining intensities of the molecular layer were also analyzed, but this data was omitted for reasons explained in the discussion section. For IBA-1, the average cell body density of IBA-1 stained microglia in the

molecular and granular layers, as well as microglia in the white matter, were both separately determined. This was done, for each brain, by taking two randomly selected square areas in the white matter, as well as the molecular and granular layers, counting the number of cells in each, and dividing the count by the respective measured area to find the density. Average microglial cell body density was then compared between brains.

Genotypes for each of the mice used were verified using Polymerase Chain Reaction (PCR) and gel electrophoresis. DNA samples were extracted from ear punches and tail clippings from the mice. DNA samples were then analyzed for the presence of the Lys-M-Cre and $IKK\beta^{Flox}$ genotypes, using Lys-M-Cre and IKKβ specific primers. The researcher was blind to the genotypes of the mice until after processing the data generated by analyzing stain images.

Results:

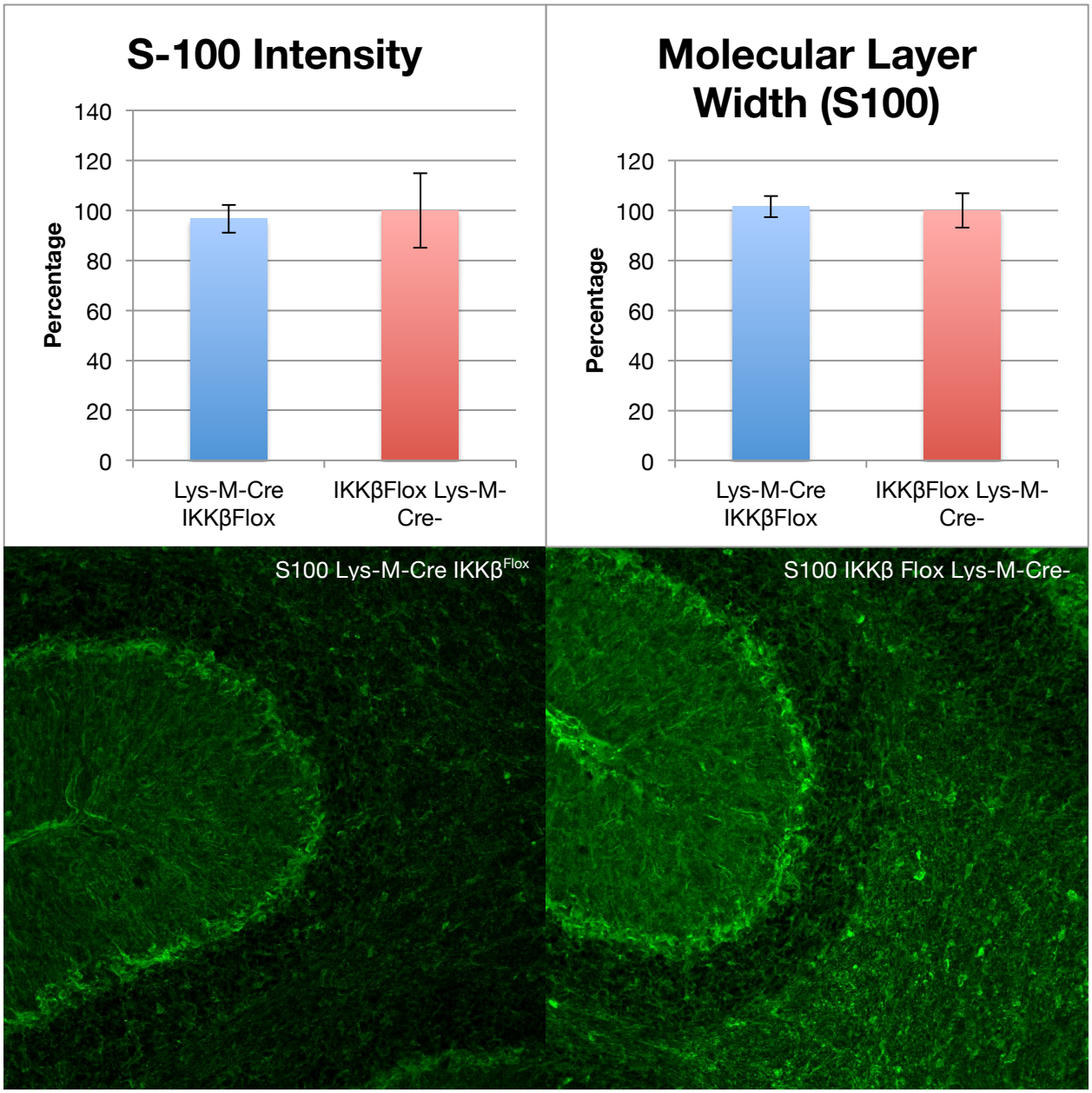

Figure 1: S-100 analysis of intensity and width of the molecular layer. Lys-M-Cre IKKβ^{Flox} mice demonstrated 3.4±5.5% lower average S-100 intensity than IKKβ Flox Lys-M-Cre-mice. The average molecular layer width, in Lys-M-Cre IKKβ^{Flox} mice, was 1.5±4.2% wider than that in IKKβ Flox Lys-M-Cre-mice. Errors for S-100 intensity and width of the molecular layer and the white matter in the IKKβ Flox Lys-M-Cre-controls are ±14.9% and ±6.9%, respectively. Errors and error bars are expressed as standard error of mean.

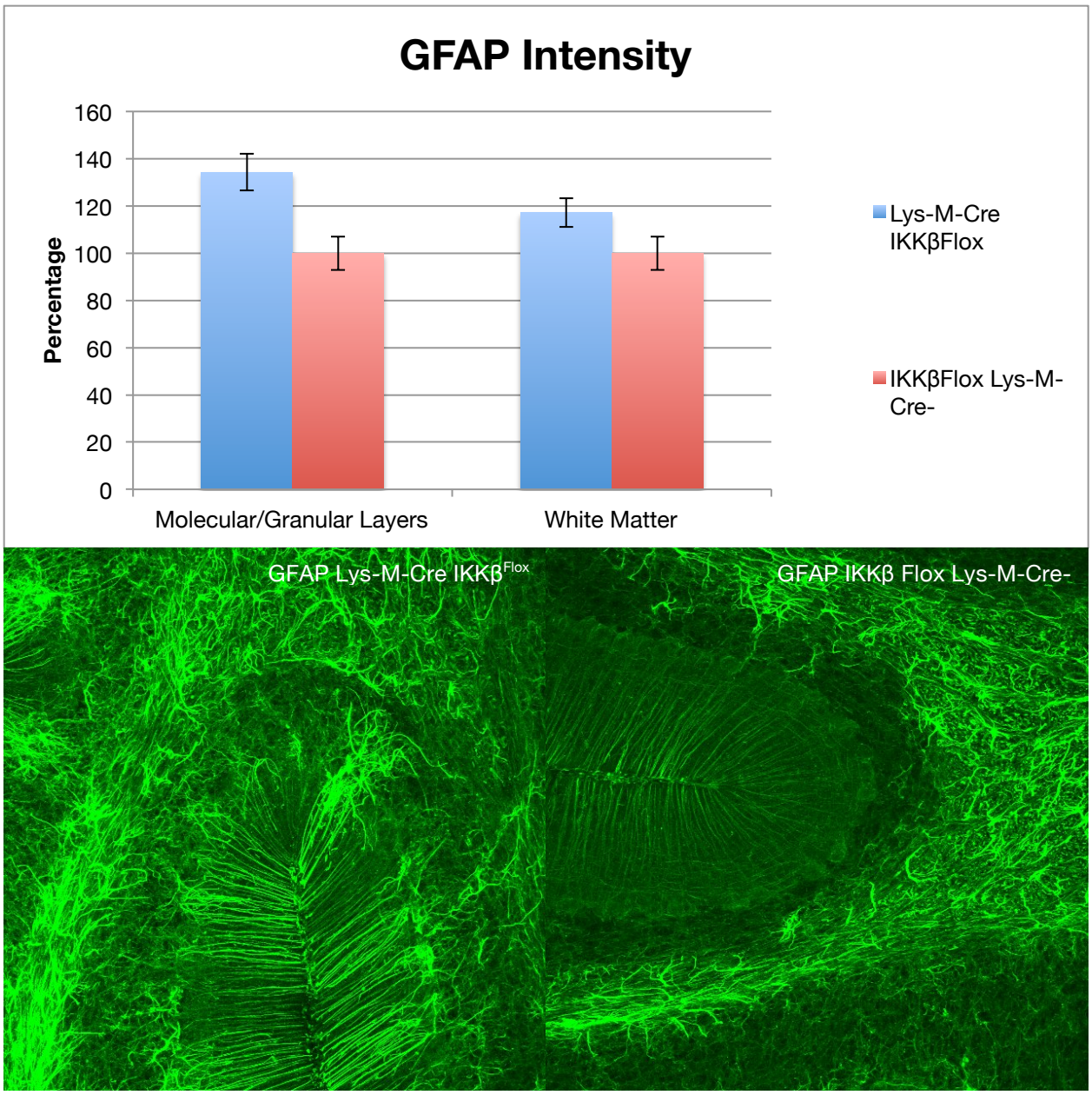

Figure 2: GFAP analysis of white matter and molecular with granular layer intensity. Lys-M-Cre IKKβ^{Flox} mice demonstrated 34.3±7.7% increased staining in the molecular and granular layers, and 17.2±6.0% increased staining in the white matter, as compared to IKKβ Flox Lys-M-Cre-. Errors of the molecular with granular layers and the white matter in control IKKβ Flox Lys-M-Cre- mice are ±7.1% for both. Errors and error bars are expressed as standard error of mean.

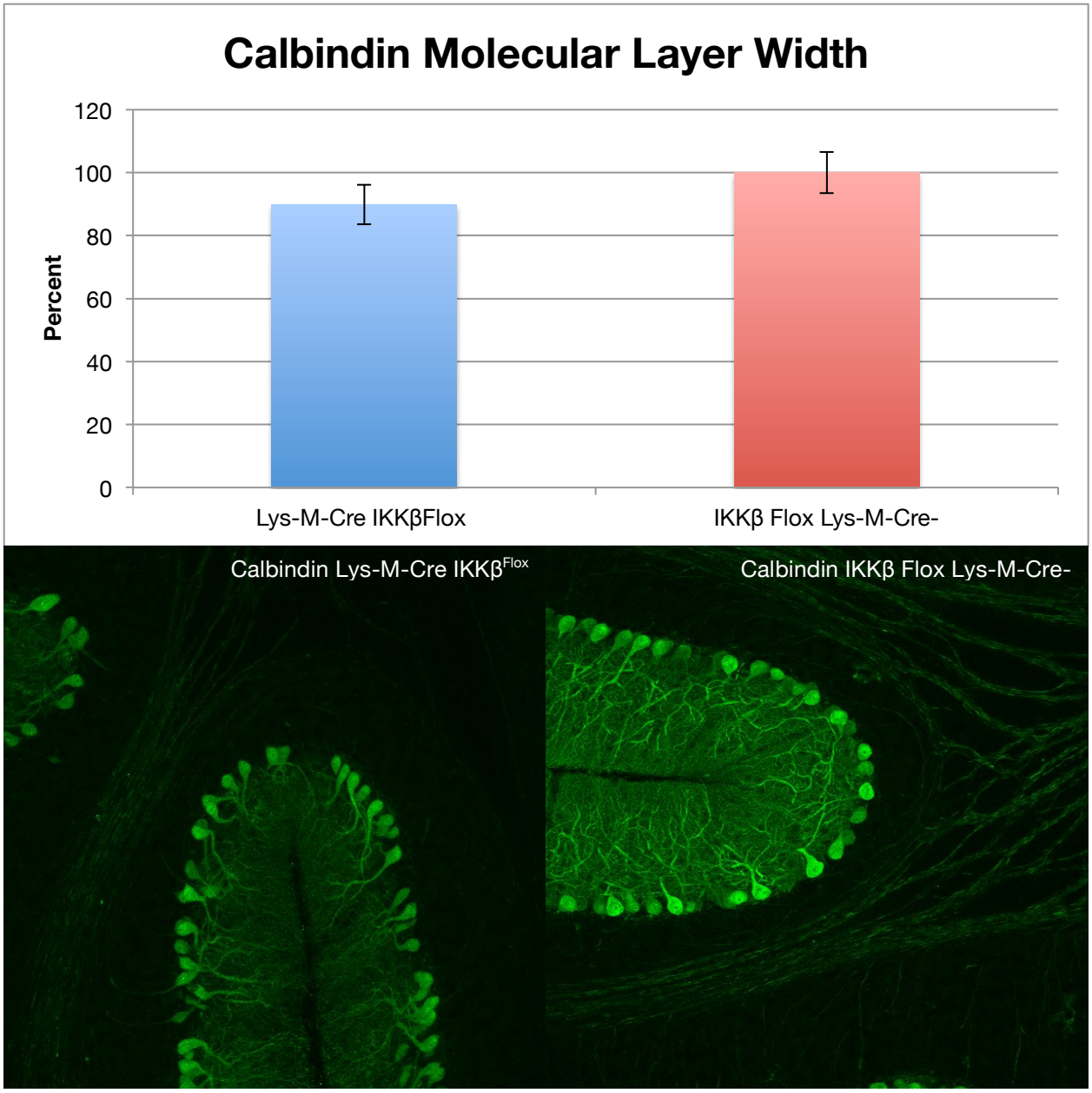

Figure 3: Calbindin analysis of molecular layer width. The molecular layer in Lys-M-Cre IKKβ^{Flox} mice was measured to be10.1±6.3% narrower than that in IKKβ Flox Lys-M-Cre- mice, on average. Error for control mice was ±6.5%. Errors and error bars are expressed as standard error of mean.

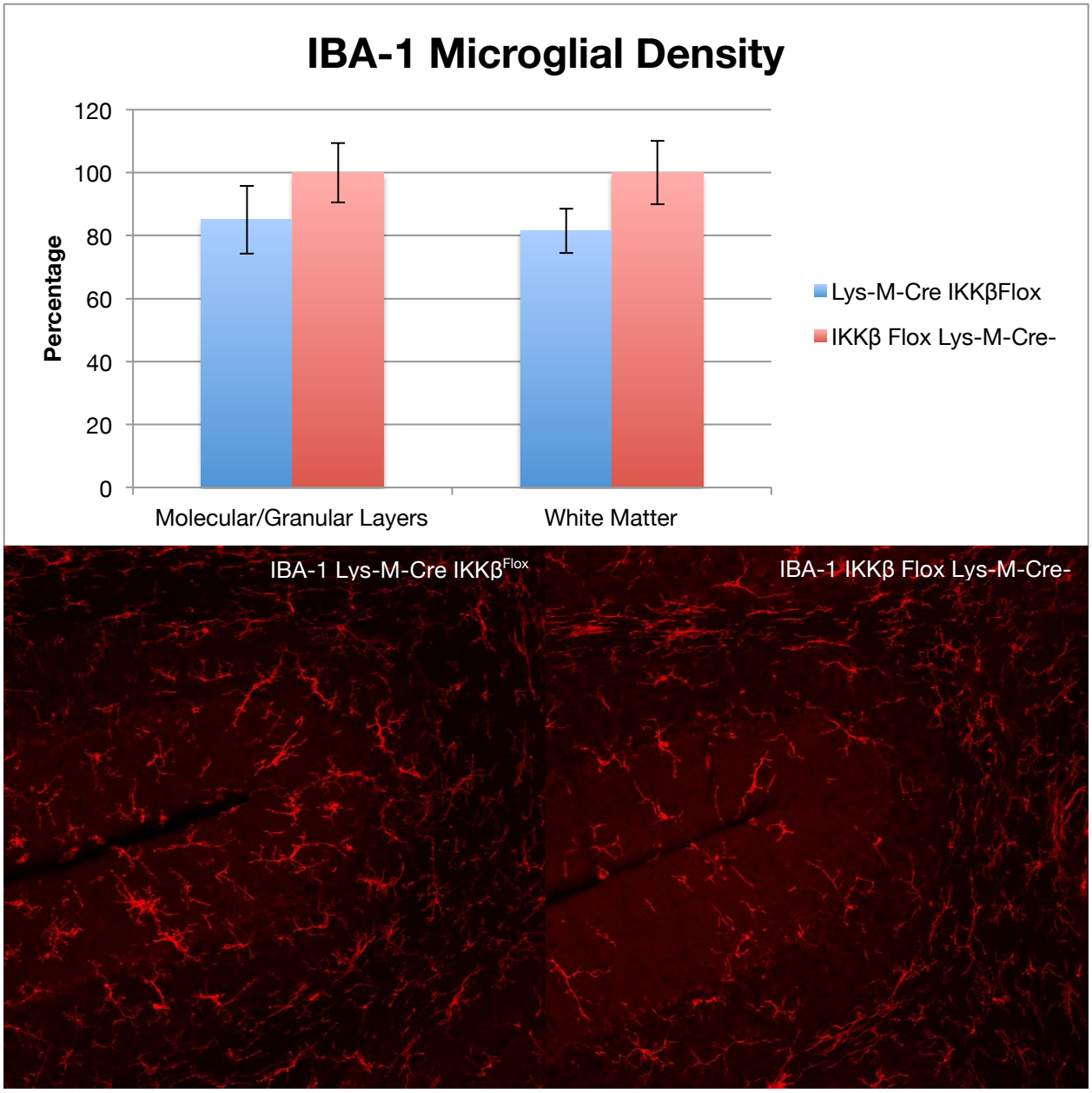

Figure 4: IBA-1 Analysis of Microglial Density. Lys-M-Cre IKKβ^{Flox} mice had an average microglial density in the molecular with granular layers 14.9±10.8% lower than IKKβ Flox Lys-M-Cre- mice, and an average microglial density in the white matter 18.45±9.5% lower than wild type. Error of the molecular with granular layers and the white matter in the IKKβ Flox Lys-M-Cre- control mice are ±9.5% and ±10.1%, respectively. Errors are expressed as a standard error of mean.

Discussion:

Collectively, the results from both stain series suggest that depleting the cytokine-mediated inflammation response in the microglia of Lys-M-Cre IKKβ^{Flox} mice has relatively little effect gliosis and cerebellar morphology. That being said, it is important to note the changes in astroglial activity detected by GFAP as well as potential effects of the Lys-M-Cre IKKβ^{Flox} modification on dendritic morphology, which are not insignificant.

IBA-1 stain intensity in Lys-M-Cre IKKβ^{Flox} mice, which directly monitored gliosis in microglia was consistently lower than IKKβ Flox Lys-M-Cre-, but not to such a significant degree that the difference surpassed error. Therefore, it could be argued that this measured reduction in gliosis is insignificant. In any case, a small decrease of gliosis in the Lys-M-Cre IKKβ^{Flox} mice is not surprising, especially if one argues that the microglia have a level of baseline activity detected by IBA-1 staining, and that the depletion of the cytokine-mediated inflammation response causes a minimal reduction in this activity.

Consistent with IBA-1, S-100 staining showed insignificantly decreased intensity for Lys-M-Cre IKKβ^{Flox} mice as compared to IKKβ Flox Lys-M-Cre-. GFAP staining showed significant increases in intensity for Lys-M-Cre IKKβ^{Flox} mice as compared to IKKβ Flox Lys-M-Cre-, in both the molecular with granular layers, and the white matter. The discrepancy between GFAP and S-100 staining is understandable: GFAP staining is a definitive measure of astrogliosis because GFAP is expressed in the cytoskeleton, while S-100 is a calcium binding protein expressed during neurological damage, and S-100 expression may not be directly proportional to astrocyte activation. The increase in GFAP staining could be explained by simple operational error, but the standard error of mean for GFAP staining is significantly lower than many other stains, like IBA-1. For this to have happened the operational error that occurred must have happened consistently, in such a manner that it only affected Lys-M-Cre IKKβ^{Flox} mice. This is unlikely, since all tissue samples were stained together, and the researcher performing the staining and analysis was double blind to the genotypes. A more likely explanation is that something about the Lys-M-Cre IKKβ^{Flox} modification caused this change. One possible theory put forth is that the astrocytes could be more activated in Lys-M-Cre IKKβ^{Flox} mice in response to a need for synaptic pruning. Normally, microglia engulf excess synapses, which express fractalkine. If the Lys-M-Cre IKKβ^{Flox} modification interferes with the ability of microglia to engage in synaptic pruning, the astrocytes activation could increase to make up for the failure of microglia to engage in synaptic pruning, which would explain the increased GFAP staining. This is possible considering that astrocytes actively participate in synaptic pruning through the MEGF10 and MERTK pathways (Chung et al., 2013). These ideas merit more GFAP analysis of the cerebella in Lys-M-Cre IKKβ^{Flox} mice in order to provide more consistent and definitive data, as well as a thorough evaluation of the theory that the astrocytes could be expressing a compensatory response in the face of the depletion of the cytokine-mediated inflammation response of the microglia.

Average width of the molecular layer, an easily measured indicator of changes of morphology, remained consistent for both S-100 and Calbindin staining. This would suggest that the Lys-M-Cre IKKβ^{Flox} modification does not significantly affect neuron development or complexity. Despite the consistent width, a wild increase in calbindin stain intensity in Lys-M-Cre IKKβ^{Flox} mice was observed. This data was omitted since staining was so increased that oversaturation occurred causing a large proportion of pixels were at the maximum possible intensity, a phenomenon that would artificially reduce standard deviation and average intensity, making this data unreliable. This dramatic increase in Calbindin staining, if not a result of

operator error, could be supportive of the theory that the Lys-M-Cre IKKβ^{Flox} modification leads to reduced synaptic pruning. Overall, the results of the intensity analysis of calbindin staining demands further investigation intothe impact of the Lys-M-Cre IKKβ^{Flox} modification on dendritic morphology of the Purkinje neurons.

Conclusion:

Despite the variations in GFAP and Calbindin staining, it is still evident that at least for microglial activity, the Lys-M-Cre IKKβ^{Flox} modification does not present significant variations from IKKβ Flox Lys-M-Cre-, making the Lys-M-Cre IKKβ^{Flox} mouse a good model for further study of the depletion of the cytokine mediated inflammation response in the microglia. Still, it is extremely important to further investigate the reasons behind the increase in GFAP staining, and if such a change could have an impact on the phenotype in a mouse model of the SCA-1 disease. This would be done first and foremost with studies similar to this one using larger sample sizes, and if further investigation is merited, dedicated analysis of synaptic pruning in Lys-M-Cre IKKβ^{Flox} mice. For now though, the next step is to apply the Lys-M-Cre IKKβ^{Flox} modification to the mouse model of SCA-1, and to evaluate the effects of depleting the cytokine-mediated inflammation response on the pathology of neurological degeneration present in SCA-1.

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